IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

APPELLANTS:	Klaus Abraham-Fuchs et al	CONFIRMATION NO. 2613
SERIAL NO.:	09/784,720	GROUP ART UNIT: 1631
FILED:	February 15, 2001	EXAMINER: C. Mahatan
TITLE:	"NETWORK FOR EVALUATING DATA OBTAINED IN A BIOCHIP MEASUREMENT DEVICE"	

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APPELLANTS' APPEAL BRIEF

S I R:

In accordance with the provisions of 37 C.F.R. §41.37, Appellants herewith submit their main brief in support of the appeal of the above-referenced application.

REAL PARTY IN INTEREST:

The real party in interest is the assignee of the present application, Siemens Aktiengesellschaft, a German corporation.

RELATED APPEALS AND INTERFERENCES:

There are no related appeals and no related interferences.

STATUS OF CLAIMS:

Claims 2-8 and 10-18 are the subject of the present appeal, and constitute all pending claims of the application. Claims 2 and 9 have been cancelled.

STATUS OF AMENDMENTS:

No Amendment was filed following the final rejection.

SUMMARY OF THE INVENTION:

The subject matter of the claims on appeal provides a cost-efficient and clinically reliable way of obtaining clinical measurement and diagnosis data for a new

diagnostic marker or a new multi-marker expert rule, which is suitable for collecting a volume of data sufficient to support an approval procedure for the marker or rule with a national health authority, such as the FDA. (p. 2, l. 13-17)

The present invention makes use of currently available biosensor arrays (biochips) and Electronic Patient Records (EPR). (p. 4, l. 14-15)

A new generation of biosensor arrays has been developed and is about to enter widespread use in the medical diagnostic market. Instead of conducting multiple measurements of multiple markers with a number of different devices, or using highly sophisticated robots in a centralized diagnostic laboratory, the new generation of biosensor arrays are able to measure, in a fully automated manner, a large number of markers simultaneously, up to thousands of different markers on the same chip, without a need for further human interaction. (p. 4, l. 16-22) Moreover, such measurement are made outside of a formal laboratory environment. Almost all known types of biomolecular markers (e.g. DNA fragments, proteins, enzymes, antibodies, etc.) can be measured simultaneously on the same chip. These biochips are particularly suited for immediately conducting the diagnostic test at a point of care (POC) site, such as a hospital bedside, a physician's office, or even at the patient's home. Such biochips also, of course, can be used in a professional centralized laboratory. (p. 4, l. 22 – p. 5, l. 4)

As shown in Figure 1, the inventive apparatus includes a biochip measurement device, for use with a number of multi-parameter biochips. By means of known measurements in the biochip measurement device an EPR segment 1 containing biochip data is produced for each of a number of patients (patient 1, patient 2, patient 3, etc.). (p. 5, l. 17-21)

The apparatus also includes a user interface for entering medical diagnostic data, collectively referred to herein as clinical data, which includes diagnostic data and/or patient history data. Through an electronic connection, the user interface produces, for each patient, an EPR segment 2 containing the diagnostic and/or patient history data. (p. 5, l. 22, p. 6, l. 2)

The EPR segments 1 and the EPR segments 2 are electronically transmitted to a database in a central server, wherein they are stored. The central server also includes a unit for evaluation and testing of the information in the database, according to algorithms for performing statistical analysis. (p. 6, l. 3-6) The central server is connected to a user interface, at which expert rules, such as for a measurement protocol for a selected pathology, are displayed as a result of the evaluation and testing conducted in the central server. The user interface also makes available information regarding biochip test sensitivity and specificity, and if necessary, documentation for approval of the measurement protocol. (p. 6, l. 6-11)

Through another electronic connection, the expert rule can be modified. (p. 6, l. 12)

Figure 2 shows an example of an information exchanges for explaining the manner by which such biosensor arrays and EPRs are used in the inventive network and procedure, assume that a diagnostic test for a certain disease, such as cervical cancer, using five different biomolecular markers, is approved and is regularly practiced in the daily routine in a physician's office to diagnose women with the suspicion of developing this type of cancer, or a screening tool for women who may be at risk from a certain age to develop cervical cancer. (p. 6, l. 13-19) A "cervical cancer biochip" is then available for conducting all of the diagnostic tests which are a

part of the approved routine for the five markers. For each patient, a disposable biochip with an appropriate sample from the patient is obtained, and the disposable chip with the patient sample is inserted into a suitable point of care test device, such as at the physician's office (step 1 in Figure 2). (p. 6, l. 19-24) The diagnostic test is conducted, possibly with the point of care test device requesting measurement protocols for conducting the tests via a communication link with a remote server (step 2 in Figure 2). (p. 6, l. 24 – p. 7, l. 2) If such a request is made, the remote server, from a data bank of measurement protocols, selects the appropriate measurement protocol and transmits it back via the data link to the point of care test device (step 3 in Figure 2). (p. 7, l. 2-5) Of course, if the protocol is conducted often enough at the point of care test device, the protocol can be stored in the device itself, in which case there is no need to establish communication at the time of the test with the remote server. As used herein a "protocol" not only specifies a procedure, but also the markers which are to be considered in the procedure. (p. 7, l. 5-9)

The results of the diagnostic test conducted using the "cervical cancer biochip" are entered into and stored in the EPR of the patient, which is accessible at the point of care site via a data entry station. (p. 7, l. 10-12) Since no medical diagnostic test can be unequivocally stated to have a 100% accuracy, there will always be the possibility of a false positive result or a false negative result. (p. 7, l. 12-14) In false positive cases, the patient (as a result of the false positive diagnosis) will be referred to a clinic for further evaluation, such as for conducting a biopsy. (p. 7, l. 14-16) The biopsy analysis will show that there is, in fact, no cancer present, and this will also be indicated in the patient's EPR. (p. 7, l. 16-17) In false negative cases, i.e., where an existing cancer is not diagnosed by the biochip, there will come

a time within weeks or months wherein the patient will, in fact, be diagnosed to have cervical cancer, and such a diagnostic entry will be made in the patient's EPR. (p. 7, l. 17-20) Thus, over time, every EPR will contain a data entry such as "biochip measurement result" and a follow-up entry (in some form) "cervical cancel diagnosis: positive or negative". Every EPR, therefore, will contain an indication of the correctness of the biochip measurement result which, in turn, is an indication of the efficacy of the protocol used to analyze the biochip data. Automated evaluation of the EPR information is thus able to yield quantified outcome data for the specificity and sensitivity of the "cervical cancer biochip" test under consideration. (p. 7, l. 20 – p. 8, l. 3)

As almost always occurs, however, assume that medical progress results in new and possibly more sensitive or more specific markers being identified for cervical cancer. These new markers can be implemented in the context of existing, approved biochip test, as an augmentation in addition to testing for the established markers, and measurement results can be simultaneously obtained. (p. 8, l. 4-8) The new data are not included in making the diagnostic decision according to the approved protocol, but nevertheless are still stored in the EPR. Since the data are measured and are available together with the final diagnostic result, any hypothesis as to improvement of the sensitivity or specificity can be retrospectively tested, in the same manner described above for the approved protocol. (p. 8, l. 10-14) The hypothesis may be, for example, that the additional markers increase performance, or that one marker can replace a less indicative approved marker in the test. The data will establish the basis for final approval of an improved test by a regulatory authority. (p. 8, l. 15-17) By such a procedure, a continuous improvement in multi-

marker tests is achieved, at virtually no additional cost, using clinical procedures which are already being conducted in any event for the approved procedure. Improved diagnostic markers can thus be developed in a very cost-effective manner. (p. 8, l. 18-21)

This is indicated in Figure 2 in step 4, wherein the measurement is conducted at the point of care testing device using the approved markers as well as the aforementioned "hidden" markers. (p. 8, l. 22-24) The raw point of care data obtained as a result of this measurement are transmitted to the remote server, particularly to a data evaluation expert system at, or accessible by, the remote server. (p. 8, l. 24 – p. 9, l. 2) The expert system applies expert rules to obtain an evaluation result (diagnosis) in step 6, with this diagnosis result being transmitted back to the point of care site. (p. 9, l. 3-5) The diagnosis result can be displayed, in step 7, at the point of care device. The displayed result, however, will be only at this time for the approved test, but the expert system at the remote server can use the totality of the data (i.e., data relating to approved markers as well as "hidden" markers) to execute an appropriate learning procedure so as to adjust or modify the evaluation rules. (p. 9, l. 5-10)

Extending this scenario, even diagnostic tests for new diseases can be developed very cost-effectively. For example, assume there exists no currently approved tests for ovarian cancer, but several markers which are indicative of this disease are suggested. (p. 9, l. 11-14) Measurement of these markers can be done in the same sample as is used in the "cervical cancer biochip", i.e., a cervical swab. The proposed set of markers for ovarian cancer then can be employed as the "hidden" markers on the chip, and data relating thereto can be measured and stored

automatically in the "background" of each cervical cancer test. (p. 9, l. 14-18) If any of the women develops ovarian cancer, this will be diagnosed at a later time, and this diagnosis will be entered into the patient's EPR, and retrospectively correlated with the "hidden" biochip test. By collecting such data over a large number of patients, final regulatory approval for an ovarian cancer test can thus be obtained. (p. 9, l. 18-22).

The automated, retrospective correlation of biochip measurement data and medical diagnosis in the EPR can also serve to gradually and automatically improve the expert rule for evaluation of a multi-parameter biochip test. (p. 9, l. 23-25) An expert rule developed with available data from 500 patients may be improved if optimized based on data from 1,000 patients or 10,000 patients. (p. 9, l. 25 – p. 10, l. 2) A browser can automatically evaluate the increasing data base in the various EPRs from an increasing number of patients over predetermined time intervals, such as by using self-learning algorithms in the manner of a neural network to improve the evaluation rules. (p. 10, l. 2-5)

GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL:

The following issues are presented for review in the present appeal:

Whether claims 2-8 and 10-18 comply with the written description requirement of 35 U.S.C. §112, first paragraph;

Whether claims 2-8 and 10-18 comply with the enablement requirement of 35 U.S.C. §112, first paragraph; and

Whether claims 2-8 and 10-18 satisfy the requirement to be definite under 35 U.S.C. §112, second paragraph.

ARGUMENT:

"Written Description" Rejection Of Claims 2-8 And 10-18 Under 35 U.S.C. §112, First Paragraph.

In the final rejection made in the Office Action dated July 6, 2005, all of the claims 1-8 and 10-18 were rejected under 35 U.S.C. §112, first paragraph as failing to comply with the written description requirement. The Examiner also made the statement that claims 17 and 18 (which were newly added in Appellants' response immediately preceding the final rejection) and the amendments to claims 2-8 and 10-16 (made in that same response) are considered by the Examiner to be new matter. This is consistent with the requirement of *In re Rasmussen*, 650 F.2d 1212, 211 U.S.P.Q. 323 (CCPA 1981), and the requirements in MPEP 2163.06 based thereon. Accordingly, as also set forth in MPEP 2163.06, the new matter rejection of the claims, based on a failing to satisfy the written description requirement, is reviewable by appeal, rather than by petition. Moreover, for the purpose of deciding this issue, the law relating to satisfaction of the written description requirement should be applied in view of the statement by the Federal Circuit in *Moba, B.V. v. Diamond Automation, Inc.*, 325 F.3d 1306, 1319, 66 U.S.P.Q. 2d 1429 (Fed. Cir. 2003), *cert. denied*, 540 US 982 (2003), commenting on the *In re Rasmussen* decision, that

> [T]his Court's predecessor explained that the use of §132 or §112 was synonymous because "a rejection of an amended claim under §132 is equivalent to a rejection under §112, first paragraph."

As a starting point, it is clear that whether a specification complies with the written description requirement is a determination of fact. *In re Curtis*, 354 F.3d 1347, 1352, 69 U.S.P.Q. 2d 1274 (Fed. Cir. 2004); *Chen v. Bouchard*, 347 F.3d 1299 1304-1305, 68 U.S.P.Q. 2d 1705 (Fed. Cir. 2003). The same is true for

assessing whether the claims satisfy the written description requirement in the context of a new matter rejection. Like any other question of fact, therefore, the Board must determine whether the evidence presented by the Appellants represents a preponderance in the context of the facts that are relevant to determining when the written description requirement has been satisfied. On this point, hundreds if not thousands of decisions have stated that the subject matter of a claim in question need not be described literally or "i*n haec verba*" in order for the specification to satisfy the description requirement. *In re Lukach*, 442 F.2d 967, 969, 169 U.S.P.Q. 795 (CCPA 1971). It is sufficient that the specification "convey clearly to those skilled in the art, to whom it is addressed, in any way, the information that the Applicant has invented the specific subject matter later claimed." *In re Wertheim*, 541 F.2d 257, 262, 191 U.S.P.Q. 90, 97 (CCPA 1976), *appeal after remand*, 646 F.2d 527, 209 U.S.P.Q. 554 (CCPA 1981). As stated by the Federal Circuit in *In re Daniels*, 144 F.3d 1452, 1456, 46 U.S.P.Q. 2d 1788, 1790 (Fed. Cir. 1998)

> In general, precedent establishes that although the Applicant "does not have to describe exactly the subject matter claimed, the description must clearly allow persons of ordinary skill in the art to recognize that [the Applicant] invented what is claimed." *In re Gosteli*, 872 F.2d at 1012, U.S.P.Q. 2d at 1618.

The last-cited decision of *In re Gosteli*, 872 F.2d 1008, 1012, 10 U.S.P.Q. 2d, 1614, 1618 (Fed. Cir. 1989) stated

> [T]he test for sufficiency of support in a patent application is whether the disclosure of the application relied upon "reasonably conveys to the artisan that the inventor had possession at that time of the later claimed subject matter.

In the final rejection, the Examiner stated that the basis for the rejection under §112, first paragraph of claims 2-8 and 10-18 is that claims 7, 17 and 18 that the biochips "are sensitive for more biomolecular markers than said predetermined

number of biomolecular markers is not supported in the disclosure. The Examiner also stated the language in claims 7 and 17 referring to the use by the evaluation system of "all of said follow-up diagnostic data as a training data set" is not supported in the original disclosure.

Applying the above standards from the relevant decisions for assessing this question of fact, Appellants should prevail if they demonstrate, by a preponderance of the evidence (i.e., Appellants' position is slightly more likely than the Examiner's position) that the original disclosure conveys to a person of ordinary skill in the art that the Appellants were in possession of both of the aforementioned claim language features.

Appellants acknowledge that the word "sensitive" is not specifically used in the original disclosure, however, as the above case law makes clear, this is not unusual and is by no means dispositive of a determination as to whether the written description requirement is satisfied. The original disclosure includes the language identified above (in the Summary Of Claimed Subject Matter) at page 4, lines 16-22 of the original specification. That passage states that instead of conducting multiple measurements of multiple markers with a number of different devices, or using highly sophisticated robots in a centralized diagnostic library, a new generation of biosensor arrays will be used, that are able to measure, in a fully automated manner, a large number of markers *simultaneously*, up to thousands of different markers on the same chip, without a need for further human interaction. Saying, as in the present claim language, that each biochip is sensitive for multiple biomolecular markers, is clearly described by this passage in the original disclosure, at least to the extent necessary to convey to a person of ordinary skill in the relevant technology

that the Appellants were in possession of that subject matter. Moreover, as clearly indicated in the present specification, the biochips that are sensitive for a multiple biomolecular markers are commercially available items, which those of ordinary skill in the relevant technology know to be commercially available, and therefore referring to those items in the manner described in the original specification, as opposed to the manner used to describe those items in the claims, makes absolutely no difference to those of ordinary skill in the art. Elsewhere in the final rejection (at page 8) the Examiner provided a dictionary definition of "sensitive" as meaning "...readily effected or changed by various agents (as light or mechanical shock)", and Appellants have no reason to disagree with that definition. Appellants submit this is exactly what is being described in the aforementioned passage in the original disclosure in terms of biosensor arrays that are able to measure a large number of markers simultaneously. Clearly in order to measure a marker, the biochip must be "sensitive "to that marker.

Additionally, the original specification (also cited above in the Summary Of Claimed Subject Matter) at page 8, line 2 refers to "outcome data for the specificity and sensitivity of the "cervical cancer biochip" test under consideration. The Examiner also provided a dictionary definition of "sensitivity" as "...the quality or state of being sensitive," and therefore if a biochip has a "sensitivity" this necessarily means it must be "sensitive" to something, in this case a plurality of biomolecular markers.

As to the phrase "said follow-up diagnostic data as a training data set," it should be noted that this phrase is part of a larger phrase describing a function of the evaluation system of "using all of said point of care raw data and all of said follow-up

diagnostic data as a training data set." A "training data set" is a term of art well known to those of ordinary skill in the use of expert systems (neural networks). A training data set is used to train the expert system so that the expert system automatically performs a more refined analysis of the next-incoming set of input data, by virtue of being "trained." Each of claims 17 and 18 explicitly refers to an expert system to which the raw point of care data is entered, as an input, for producing a diagnostic result using an expert rule. As is well known to those of ordinary skill in the field of expert systems and neural networks, such an expert system or neural network is "trained" by using data to modify one of the rules used by the system or network. This is standard terminology that is used in many of the references of record, including the Mendoza et al reference (discussed in more detail below).

The use of the data for this purpose, as training data, is clearly described in the specification as originally filed in the paragraph bridging pages 9 and 10 of the original specification, also cited above in the summary of the claimed subject matter. This language states that the automated, retrospective correlation of biochip measurement data and medical diagnosis in the EPR (electronic patient record) can serve to gradually and automatically improve the expert rule for evaluation of a multi-parameter biochip test. This passage further state that a browser can automatically evaluate the increasing data base in the various EPRs from an increasing number of patients over predetermined time intervals, such as by using self-learning algorithms in the manner of a neural network to improve the evaluation rules.

This passage clearly refers to training a neural network by modification of the expert rules thereof, and thus clearly conveys to a person of ordinary skill in the

relevant technology that the Appellants were in possession of this feature of claims 17 and 18 at the time the original application was filed.

Both of these claim language phrases, therefore, are adequately described in the original disclosure in compliance with §112, first paragraph, and thus their inclusion in claims 7, 17 and 18 does not justify a rejection of claims 2-8 and 10-18 as containing new matter.

Rejection Of Claims 2-8 And 10-18 For Lack Of Enablement Under 35 U.S.C. §112, First Paragraph

As briefly noted above, an important feature of the subject matter of the claims on appeal is the use of the data gathered from biochips from multiple patients to modify an expert rule used by an expert system to produce a diagnostic result. As best as Appellants can understand the lack of enablement rejection of claims 2-8 and 10-18, it is based on the position of the Examiner that the original disclosure does not provide information as to how the gathered data are used for that purpose. Specifically, the Examiner believes that some type of standard must be presented against which a determination can be made as to whether an improvement in the diagnosis has occurred.

As Appellants have previously argued, the details of the manner by which the expert system makes uses of the gathered data to produce a modified expert rule, and therefore (it is hoped) an improved diagnostic result, form no part of the present invention. The present invention is based on the insight that the huge amount of data that are collected during the normal course of the use of biochip measurement devices for a large patient population can be advantageously supplied as training data to an expert system. What happens within the expert system after the data are supplied thereto is of consequence of the present invention only because (naturally)

it is hoped that the inventive data compilation will result in an improvement in the output of the expert system (neural network). The only reason why the term "modified expert rule" is used in the claim language is to differentiate it from the original rule that existed before the input of the training data. There is no "standard" for the actual rule itself, the only yardstick is whether the overall operation of the expert system, does, in fact, result in an improved diagnostic result. This obviously cannot be determined from one individual case to the next, and therefore there is no single "standard" that can be employed to compare one diagnostic result with regard to the next-successive diagnostic result. Improvement in the diagnostic capability of an expert system must be monitored over time, and in the medical field it is easily ascertainable whether such an improved diagnostic result or trend is occurring. In the example given in the present specification for detecting cervical cancer, it is known with certainty whether the diagnosis ultimately is correct or incorrect for each patient. If an improvement in the total number of correctly diagnosed patients (or, conversely, a decrease in the number of incorrectly diagnosed patients) is ascertained in a large patient population over time, this is all the information that is necessary to determine whether the expert system is really being diagnostically improved by the training data. As with any result that is statistically dependent, the available amount of data used to evaluate success or non-success changes every time a new input is made to the expert system, and therefore it is impossible to try to set a "standard' by which success or non-success can always be evaluated. Success within a patient population of one hundred patients may be considered quite differently from success within a patient population of ten thousand patients. Those

of ordinary skill in this field, however, are perfectly capable of making such an assessment, taking all of these factors into account.

As stated by the Federal Circuit in *In Re Vaeck*, 947 F.2d 488, 495, 20 U.S.P.Q. 2d 1438, 1444 (Fed. Cir. 1991)

> The first paragraph of 35 U.S.C. §112 requires, *inter alia*, that the specification of a patent enable any person skilled in the art to which it pertains to make and use the claimed invention. Although the statute does not say so, enablement requires that the specification teach those in the art to make and use the invention without "undue experimentation."...That *some* experimentation may be required is not fatal; the issue is whether the amount of experimentation required is "undue."

As stated in *AK Steel Corp. v Sollac*, 344 F.3d 1234, 1244, 68 U.S.P.Q. 2d 1280 (Fed. Cir. 2003):

> [A]s part of the *quid pro quo* of the patent bargain, the Applicant's specification must enable one of ordinary skill in the art to practice the full scope of the claimed invention...That is not to say that the specification itself must necessarily describe how to make and use every possible variant of the claimed invention, for the artisan's knowledge of the prior art and routine experimentation can often fill gaps, interpolate between embodiments, and perhaps even extrapolate beyond the disclosed embodiments, depending upon the predictability of the art.

Lastly, as stated in *Amgen, Inc. v. Hoechst Marion Roussel, Inc.*, 314 F.3d 1313, 1334, 65 U.S.P.Q. 2d 1385 (Fed. Cir. 2003)

> The specification need not explicitly teach those in the art to make and use the invention; the requirement is satisfied if, given what they already know, the specification teaches those in the art enough so that they can make and use the invention without "undue experimentation.

The Examiner at page 6 of the final rejection stated that if Appellants never intended such criteria or parameters relating to the modification of the expert rules to be embraced by the claim language or included as a part of the invention, then it is unclear why Appellants have included such language. As noted above, the aforementioned language was included in the claims solely to describe a result that

is accomplished by the inventive use of the collected data in accordance with the invention, but the expert system that is used, and thus the expert rules that are employed in that expert system, can be any expert system which is within the capability of being designed by those of ordinary skill in the art. The expert system disclosed in the aforementioned Mendoza et al article is an example. Appellants respectfully submit the Examiner is mistaken in suggesting that Appellants should be able to state, for that specific expert system, in the Mendoza et al article, some sort of standard or criteria. Appellants respectfully submit the opposite use should be made of the Mendoza et al article, namely the Mendoza et al article should be used as an example of the knowledge possessed by those of ordinary skill in the field of expert system use and design, and therefore should be used as evidence that such a person would have no difficulty, after having read the present specification, in making use of the expert system disclosed in Mendoza et al for the purposes described in the present specification.

Rejection Of Claims 2-8 And 10-18 Under 35 U.S.C. §112, Second Paragraph As Being Vague And Indefinite

Claims 7 and 17 were rejected as being vague and indefinite because of the aforementioned phrase stating "said biochips are *sensitive* for more biomolecular markers than said predetermined number of biomolecular markers..." and "A plurality of disposable biochips, each *sensitive* for multiple biomolecular markers...". Appellants submit the same arguments relating to the dictionary definitions of "sensitive" are applicable to this rejection as well. Again, however, the Examiner appears to believe that some specified criterion must be provided to enable a determination to be made as to whether a biochip is "sensitive" to a particular marker. As argued above, Appellants respectfully submit the Examiner is

overcomplicating this issue. The term "sensitive" is being used in the present specification in no more complicated a manner than in the sense that a thermometer is "sensitive " to temperature. This is so easily determinable as to be trivial. A sensor merely needs to be exposed to the marker, quantity or property in question, and either the sensor does or does not produce an output signal. If it does produce an output signal, it is "sensitive" to that marker, quantity or property.

Since Appellants are using this term entirely consistently with its dictionary definition, it is not a vague or indefinite term.

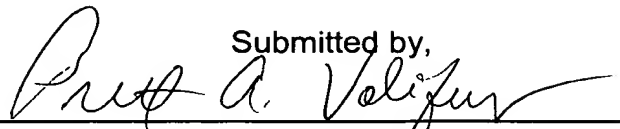
A further basis for the "vague and indefinite" rejection was phrase in claim 17 of "...creating a modified expert rule with *improved* diagnostic value in comparison to said expert rule used to produce said diagnostic result". For the reasons also argued above, Appellants respectfully submit those of ordinary skill in the relevant technology are fully capable of determining when a diagnostic result in a particular context is improved with regard to another diagnostic result. Again, however, the Examiner believes that the absence of a criterion specified in the original disclosure makes this a vague and indefinite term. Such definitive and absolute criteria, however, are not absent from the specification because they do not exist, they are absent from the specification because they are too numerous to include in any meaningful manner.

CONCLUSION:

For the foregoing reasons, Appellants respectfully submit the Examiner is in error in law and in fact in rejecting claims 2-8 and 10-18 of the present application. Reversal of those rejections is proper, and the same is respectfully requested.

This Appeal Brief is accompanied by a check in the amount of $500.00 for the requisite fee.

Submitted by,



(Reg. 27,841)

SCHIFF, HARDIN LLP
CUSTOMER NO. 26574
Patent Department
6600 Sears Tower
233 South Wacker Drive
Chicago, Illinois 60606
Telephone: 312/258-5790
Attorneys for Appellant(s).

CERTIFICATE OF MAILING

I hereby certify that a copy of this correspondence is being deposited with the United States Postal Service as First Class mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450 on October 17, 2005.

BRETT A. VALIQUET

APPENDIX "A"

2. A network as claimed in claim 17 wherein said expert evaluation system uses said modified expert rule to devise a measurement protocol.

3. A network as claimed in claim 2 wherein said expert system devises said measurement protocol for a selected pathology.

4. A network as claimed in claim 2 wherein said expert evaluation system automatically devises said measurement protocol.

5. A network as claimed in claim 2 further comprising a memory containing a plurality of measurement protocols accessible by said remote server, and wherein each point of care test device accesses said memory, via said data link, to obtain a selected measurement protocol for performing said diagnostic testing.

6. A network as claimed in claim 5 wherein said measurement protocol is for a specific pathology and employs a predetermined number of said biomolecular markers.

7. A network as claimed in claim 6 wherein said biochips are sensitive for more biomolecular markers than said predetermined number of biomolecular markers, and wherein each point of care test device conducts said diagnostic testing using all of the biomolecular markers in the sample of the tested biochip to obtain augmented testing data, and wherein each point of care test device includes said augmented testing data in said raw point of care data.

8. A network as claimed in claim 17 wherein said point of care date entry stations comprise means for entering patient history data into said electronic patient record characterizing whether said diagnostic result was a false positive, a false negative or correct.

10. A method as claimed in claim 18 wherein the step of creating a modified expert rule comprises creating a modified expert rule for devising a measurement protocol.

11. A method as claimed in claim 10 wherein the step of creating a modified rule comprises creating a modified rule for a measurement protocol for a selected pathology.

12. A method as claimed in claim 10 wherein the step of creating a modified rule comprises automatically creating a modified rule for said measurement protocol.

13. A method as claimed in claim 10 comprising storing a plurality of measurement protocols in a memory accessible from said remote server, and wherein the step of performing diagnostic testing in each point of care test device comprises establishing a data communication between a point of care test device and said memory to obtain a selected measurement protocol from said memory for use in said point of care test device for performing said diagnostic testing.

14. A method as claimed in claim 13 wherein each of said measurement protocol employs a predetermined number of said biomolecular markers.

15. A method as claimed in claim 14 comprising providing more biomolecular markers in each sample than said predetermined number and wherein the step of performing diagnostic testing includes performing diagnostic testing using said selected measurement protocol and also employing additional biomolecular markers in the sample of the tested biochip, beyond said predetermined number, to obtain augmented testing data, and including said augmented testing data in said raw point of care data.

16. A method as claimed in claim 18 comprising obtaining said follow-up data by conducting a follow-up examination of the tested patient to determine follow-up data indicating whether said test result was a false positive, a false negative or correct.

17. A network for creating a modified diagnostic expert rule, comprising:

a plurality of disposable biochips, each sensitive for multiple biomolecular markers, respectively for a plurality of patients, each biochip containing a patient sample with multiple biomolecular markers;

a plurality of point of care test devices respectively at a plurality of point of care sites, each point of care test device receiving at least one of said biochips, as a tested biochip, and performing diagnostic testing on the sample in said tested biochip to obtain raw point of care data;

an expert system to which said raw point of care data is entered, as an input, for producing a diagnostic result from said expert system using an expert rule;

a plurality of electronic patient records respectively for said patients;

a plurality of point of care data entry stations respectively having access to at least one of said electronic patient records and respectively in communication with said point of care test devices, each data entry station including means for entering follow-up diagnostic data into the electronic patient record for the patient, as a tested patient, who provided the test sample in the tested biochip;

a remote server and an evaluation system accessible by said remote server;

said remote server having at least one data link to each point of care test device and each electronic patient record, for transmitting said point of care raw data of said patient and an identification of said expert rule used to produce said diagnostic result, and said follow-up diagnostic data, to said remote server; and

said evaluation system creating a modified expert rule with improved diagnostic value in comparison to said expert rule used to produce said diagnostic result, using all of said point of care raw data and all of said follow-up diagnostic data as a training data set.

18. A method for creating a modified diagnostic expert rule, comprising the steps of:

obtaining a plurality of samples respectively from a plurality of patients and storing the samples respectively in a plurality of disposable biochips, each biochip being sensitive for multiple biomolecular markers;

providing a plurality of point of care test devices respectively at a plurality of point of care sites;

respectively receiving said biochips in said point of care test devices, each as a tested biochip, and in each point of care test device performing diagnostic testing on the sample in the tested biochip to obtain raw point of care data;

entering the raw point of care data as an input to an expert system and producing a diagnostic result with said expert system using an expert rule;

providing a remote server at a location remote from said point of care sites;

supplying the raw point of care data and an identification of said expert rule from all of the point of care sites, and the follow-up diagnostic data, to said remote server; and

at said remote server, creating a modified expert rule with improved diagnostic value compared to said expert rule used to produce said diagnostic result, using all of said point of care data and all of said follow-up diagnostic data as a training data set.

FIG. 1

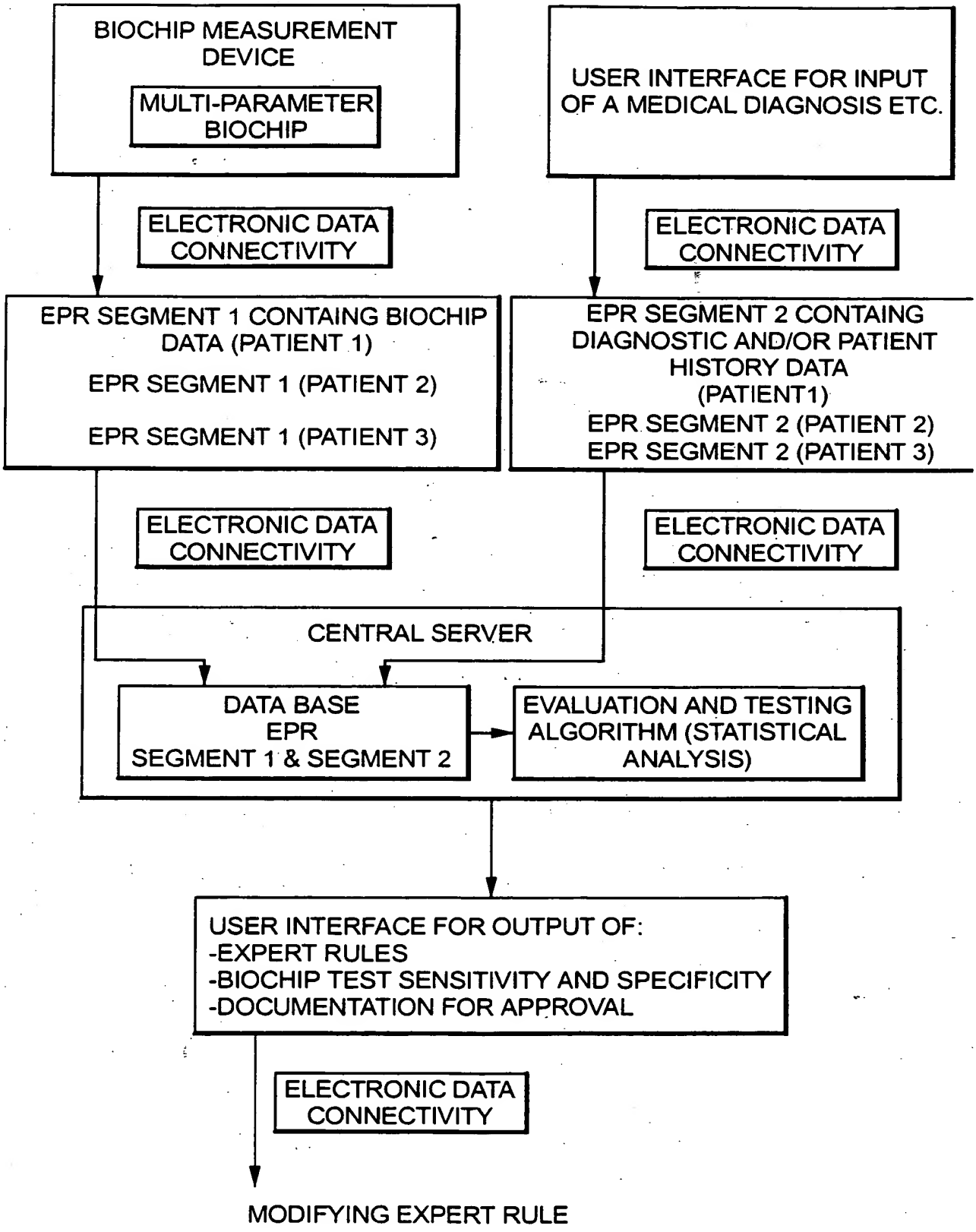
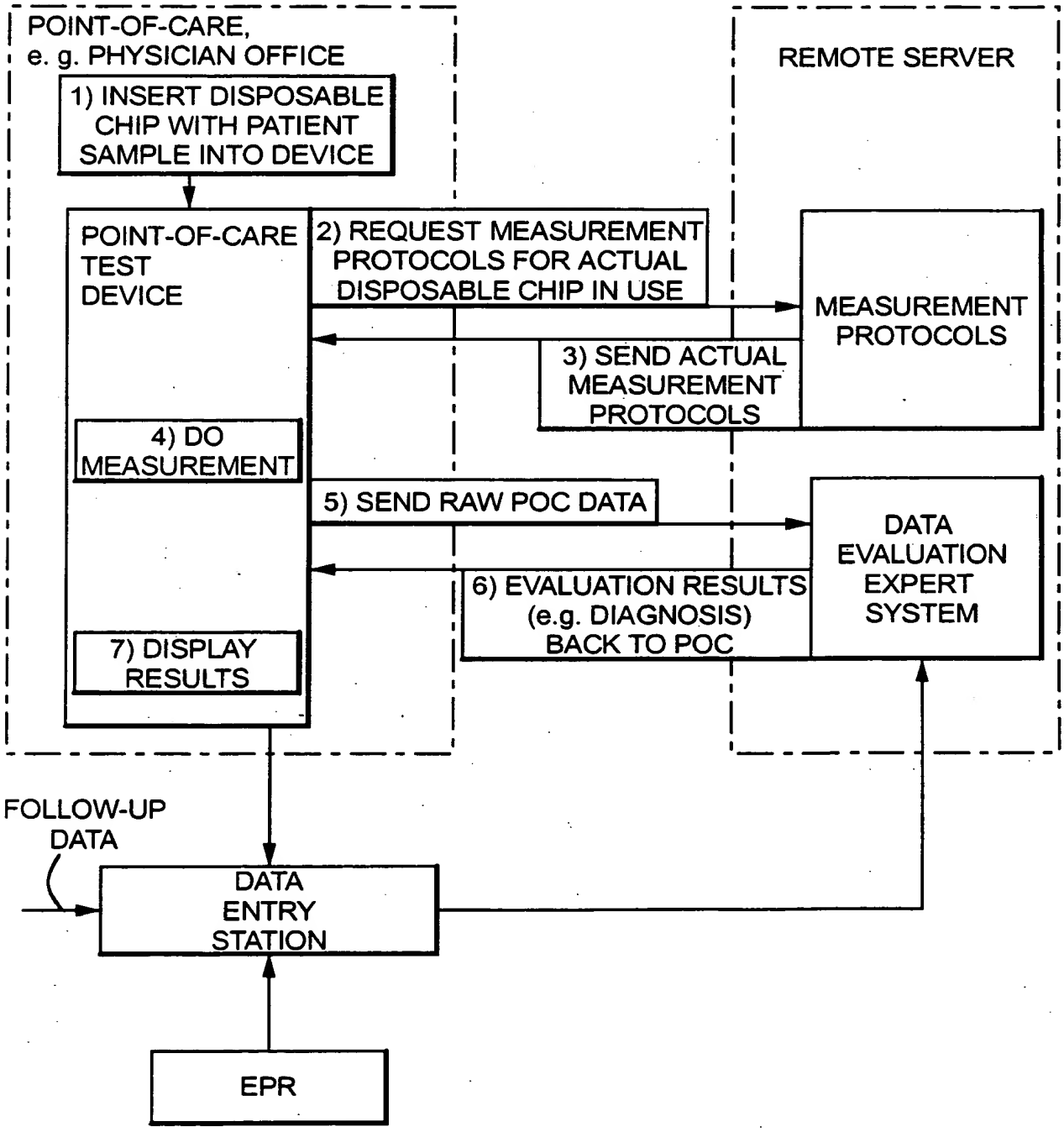


FIG. 2


POINT-OF-CARE,
e. g. PHYSICIAN OFFICE
1) INSERT DISPOSABLE CHIP WITH PATIENT SAMPLE INTO DEVICE
POINT-OF-CARE TEST DEVICE
2) REQUEST MEASUREMENT PROTOCOLS FOR ACTUAL DISPOSABLE CHIP IN USE
REMOTE SERVER
MEASUREMENT PROTOCOLS
3) SEND ACTUAL MEASUREMENT PROTOCOLS
4) DO MEASUREMENT
5) SEND RAW POC DATA
DATA EVALUATION EXPERT SYSTEM
6) EVALUATION RESULTS (e.g. DIAGNOSIS) BACK TO POC
7) DISPLAY RESULTS
FOLLOW-UP DATA
DATA ENTRY STATION
EPR

Research Report

High-Throughput Microarray-Based Enzyme-Linked Immunosorbent Assay (ELISA)

BioTechniques 27:778-788 (October 1999)

L.G. Mendoza, P. McQuary, A. Mongan, R. Gangadharan, S. Brignac and M. Eggers
Genometrix, The Woodlands, TX, USA

ABSTRACT

A new generation biochip is described as capable of supporting high-throughput (HT), multiplexed enzyme-linked immunosorbent assays (ELISAs). These biochips consist of an optically flat, glass plate containing 96 wells formed by an enclosing hydrophobic Teflon® mask. The footprint dimensions of each well and the plate precisely match those of a standard microplate. Each well contains four identical 36-element arrays (144 elements per well) comprising 8 different antigens and a marker protein. Arrays are formed by a custom, continuous flow, capillary-based print head attached to a precise, high-speed, X-Y-Z robot. The array printing capacity of a single robot exceeds 20 000 arrays per day. Arrays are quantitatively imaged using a custom, high-resolution, scanning charge-coupled device (CCD) detector with an imaging throughput of 96 arrays every 30 s. Using this new process, arrayed antigens were individually and collectively detected using standard ELISA techniques. Experiments demonstrate that specific multiplex detection of protein antigens arrayed on a glass substrate is feasible. Because of the open microarray architecture, the 96-well microarray format is compatible with automated robotic systems and supports a low-cost, highly parallel assay format. Future applications of this new high-throughput screening (HTS) format include direct cellular protein expression profiling, multiplexed assays for detection of infectious agents and cancer diagnostics.

INTRODUCTION

In recent years, DNA biochips composed of densely packed probe arrays have revolutionized genetic analysis and discovery by highly multiplexed, miniaturized, molecular assays. These tools have been used for numerous molecular assays including high-throughput (HT) genotyping (5), differential gene expression (8,9), mutation detection and DNA sequencing (1). For nearly twenty-five years, enzyme-linked immunosorbent assay (ELISA)-based immunoassays have been the mainstay of the diagnostic tests used for the detection of infectious disease. In more recent times, numerous researchers have refocused their attention on the continuing evolution of ELISA-based formats and have begun to explore miniaturized, highly multiplexed, microarray formats (2–4,6,7,10). In DNA-based arrays, high specificity is provided by Watson-Crick base pairing between complementary sequences under appropriate salt conditions using either oligonucleotide or cDNA-length probes. Alternatively, in a standard ELISA, specificity between antigen and antibody is governed by high affinity associations between an epitope on the antigen and its cognate binding site on the Fab portion of an IgG. The main challenge in developing a microarray-based ELISA is minimizing nonspecific cross-reactivity between numerous antigen and antibody mixtures, thus maintaining the integrity of the assay. In this study, we demonstrate the feasibility of simultaneously and specifically detecting numerous antigens using a 96-well, microarray-based ELISA. The specificity and sensitivity of this new ELISA format is discussed.

MATERIALS AND METHODS

All antigens and their corresponding biotin-labeled monoclonal antibodies were purchased from Sigma (St. Louis, MO, USA). Lyophilized antigens were reconstituted as recommended by the manufacturer to a concentration of 10 mg/mL in 1× phosphate-buffered saline (PBS) (pH 7.4). Biotinylated monoclonal antibodies came pre-dissolved in 0.1 M sodium phosphate (pH 7.4) containing 1% bovine serum albumin (BSA). All antibody solutions were divided into aliquots and stored at -20°C. Streptavidin-alkaline phosphatase conjugates were purchased from Boehringer Mannheim (Indianapolis, IN, USA). Blocker™ Casein in PBS was purchased from Pierce Chemical (Rockford, IL, USA). Fluorescent alkaline phosphatase substrate (ELF®) was purchased from Molecular Probes (Eugene, OR, USA). Print capillaries (100-µm diameter) were purchased from J&W Scientific (Folsom, CA, USA)

Optically flat, teflon-coated, 96-well glass microscope plates were manufactured by Erie Scientific (Portsmouth, NH, USA). Aminopropyltrimethoxysilane (APTS) was purchased from Aldrich Chemical (Milwaukee, WI, USA). Bis-sulfo-succinimidyl suberate (BS^3) was purchased from Molecular BioSciences (Boulder, CO, USA).

96-Well Microarray Plate Manufacturing

All glass plates were cleaned ultrasonically in succession with a 1:10 dilution of detergent in warm tap water for 5 min in Aquasonic Cleaning Solution (Catalog No. 21811-894; VWR, West Chester, PA, USA), multiple

Table 1. Array Antigens Consist of Purified IgGs from Various Sources

Antigens
Goat IgC
Rat IgG
Cat IgG
Human IgG_4
Bovine IgG
Rabbit IgG
Chicken IgG
Guinea Pig IgG
Biotinylated BSA

The proteins were dissolved in 50 mM carbonate buffer (pH 8.3) at 100 µg/mL and printed on the 96-well microarray plate using the capillary array printer. Additionally, a twofold serial dilution of rabbit IgG corresponding to 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL and 6.25 µg/mL was also made and printed in the same array alongside the above proteins.

rinses in distilled water and 100% methanol (HPLC grade) followed by drying in a class 100 oven at 45°C. Clean glass plates were chemically functionalized by immersing the plates in a solution of APTS (5% vol/vol in absolute ethanol) for 10 min. Plates were then rinsed in 95% ethanol, allowed to air dry and heated at 80°C in a vacuum oven for 2 h to cure. Individual amino-derivitized wells were then reacted using 40 µL of a 5 mg/mL solution of BS^3 in 1× PBS (pH 7.4) for 20 min at room temperature (RT). The *N*-hydroxysuccinimide (NHS)-activated glass surface was rinsed with distilled water and placed in a 37°C, dust-free, class 100 oven for 15 min to dry. Dry activated plates were stored desiccated for up to 6 weeks before using.

Microarray Printing

The microarray printer consists of a 36-capillary array print head mounted to a high resolution X-Y-Z positioning robot. The robot-mounted capillary print head consists of 36 capillaries precisely arranged as a square 6 × 6 array. This print head can simultaneously and precisely deliver in a continuous manner up to 36 different solutions (200 pL per spot) to any flat surface and can be expanded to 256 fluids as needed. Each 6 × 6 array occupies an area of 3.24 mm^2. Individual array elements had a diameter of approximately 275 µm with a center-to-center spacing of 300 µm.

As illustrated in Figure 2, each capillary on the print head originates from a standard 96- or 384-well plate contained within a pressurized manifold. Print solutions are first aliquoted into designated wells in the microplate and then placed inside the manifold assembly. Precise fluid delivery to the glass surface is initiated and maintained through the capillaries by a custom, computer-controlled pressure regulator. Before initiating the print process, the capillaries are primed with solution, and flow to each capillary is verified by contact printing on water-sensitive print paper (Spraying Systems, Wheaton, IL, USA). After each plate is printed, protein solutions are allowed to dry on the surface of the 96-well plate. Array quality is rapidly confirmed in each well by nondestructively imaging the dried protein-salt crystals using the high-resolution charge-coupled device (CCD) camera with ambient room-light illumination. The size, uniformity and absence/presence of crystals can be determined using this simple quality control test.

The set-up time needed to initiate a specific print job is typically 30 min. Once the printer is primed, thousands of identical arrays can be rapidly and sequentially produced using this continuous contact process. After a print run is complete, it requires approximately 20 min to flush out the capillary lines before reinitiating the system for another print job.

Depending on the type of print head mounted to the robot, this instrument is capable of printing a 16 × 16 element array every second. At these rates, approximately 3500 arrays can be printed every hour using a single robot. This high-throughput robot printer can be used to deposit DNA-, RNA- or protein-based solutions. The microarray printing process is very efficient, requiring a single milliliter of antigen solution to print more than 5 million

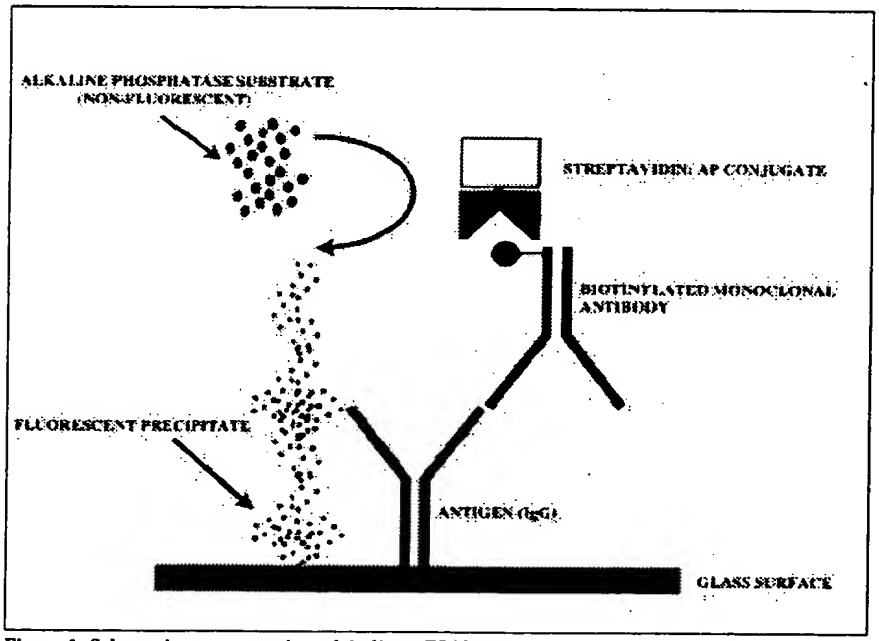


Figure 1. Schematic representation of indirect ELISA format used to detect protein arrays. The attachment substrate is optical quality glass that has been functionalized to bind proteins using a homobifunctional NHS-ester. Protein antigens consisting of purified IgGs are immobilized on the activated glass surface. Surface antigens are detected using a biotin-labeled primary monoclonal antibody specific for each antigen in the array. All primary antibody-binding events are indirectly detected using a streptavidin-alkaline phosphatase conjugate and ELF.

arrays, based upon a standard spot volume of approximately 200 pL.

Preparation of Antigen and Antibody Solutions

Each antigen stock (either 10 mg/mL or 1 mg/mL in PBS) was diluted into 50 mM carbonate buffer (pH 8.3, HCO_3^-/CO_3^{--}) to a final print concentration of 100 µg/mL. Additionally, rabbit IgG was twofold serially diluted (100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL and 6.25 µg/mL) into 50 mM carbonate buffer and printed at these varying concentrations. Print solutions were transferred into a 384-well polypropylene microplate (Corning Costar, Acton, MA, USA), and the plate was loaded inside the printer manifold. ELISAs were carried out using commercially prepared monoclonal antibodies dissolved in PBS to a final assay dilution of 1:1000 in Blocker Casein in PBS solution.

Microarray ELISA

Using an automated HYDRA® 96 Liquid Pipettor (Robbins Scientific, Sunnyvale, CA, USA), wells on the 96-well microarray plate were washed consecutively 3× with 35 µL of 1× Tris-buffered saline (TBS) + 0.1% Tween® 20 to remove excess unbound antigen. Nonspecific binding sites were blocked by pre-incubating with 50 µL of Blocker Casein in PBS for 1 h at RT. After blocking, 25 µL of the appropriate monoclonal antibody mixture (1:1000 in Blocker Casein) was incubated with an array at RT for 60 min. A single, partial or complete cocktail mixture of all 8 monoclonal antibodies was added to appropriate wells.

Following primary monoclonal antibody binding, the HYDRA 96 was used to remove excess antibody solution from each well. This was followed by washing each well 3× with 35 µL of 1× TBS + 0.1% Tween 20. Secondary detection of bound antibody complex was carried out by incubating each well with 25 µL of a 1:1000 dilution of streptavidin:alkaline phosphatase (AP) conjugate in 1× TBS + 0.1% Tween 20 for 20 min. Excess conjugate was removed, and each well was washed 3× with 50 µL of 1× TBS + 0.1% Tween 20. Array detection was initiated by adding 30 µL of the fluorescent alkaline phosphatase substrate ELF for 5 min at RT. Excess substrate was removed and wells were rinsed in distilled water. Finally, the prepared microarray plates were allowed to air dry before imaging.

Scanning CCD Imager

The main components of the custom CCD imager include: a peltier-cooled Pixel Vision camera consisting of a SpectraVideo front-illuminated CCD camera that has an attached Micro-Nikkor™ AF lens (60 mm f/2.8D; Nikon, Melville, PA, USA). The CCD captures a 1 × 1″ image region (1024 × 1024 pixels), where each CCD pixel consists of a 24-µm square area. The effective focal length of the imaging configuration is 20 cm, which maps to a calculated magnification factor of 2.16 from the CCD to the image plane. The dynamic range of the CCD imager was determined to be 1.8 logs using a stepped neutral density filter (Edmund Scientific, Barrington, NJ, USA) at a single integration time (i.e., 7.5 s). UV excitation consisted of a 6-W long wavelength (365 nm) transilluminator light source (Spectronics, Westbury, NY, USA). Motion control consists of stepper drives (New England Affiliated Technology, Lawrence, MA, USA) and Compumotor 4-axis motion control system (Parker Hannifin, Compumotor Division, Rohnert, CA, USA), all contained on a custom cart and enclosure assembled from 80/20 Parts (Columbia City, IN, USA). The system is controlled by a 333 MHz computer system (Dell Computer, Round Rock, TX, USA) with 256K of random-access memory (RAM). All other fixtures and custom parts were manufactured in-house.

The CCD is mounted to a Z-axis drive that positions the CCD camera in-line with a single microarray plate before image acquisition. The associated plate-carrier tray is mounted to an X-Y stage that provides loading/unloading and scanning of slides containing microarrays about the detection plane. The plate carrier tray accepts microarray plates held by a custom delrin insert holder that securely holds the 5 × 3″

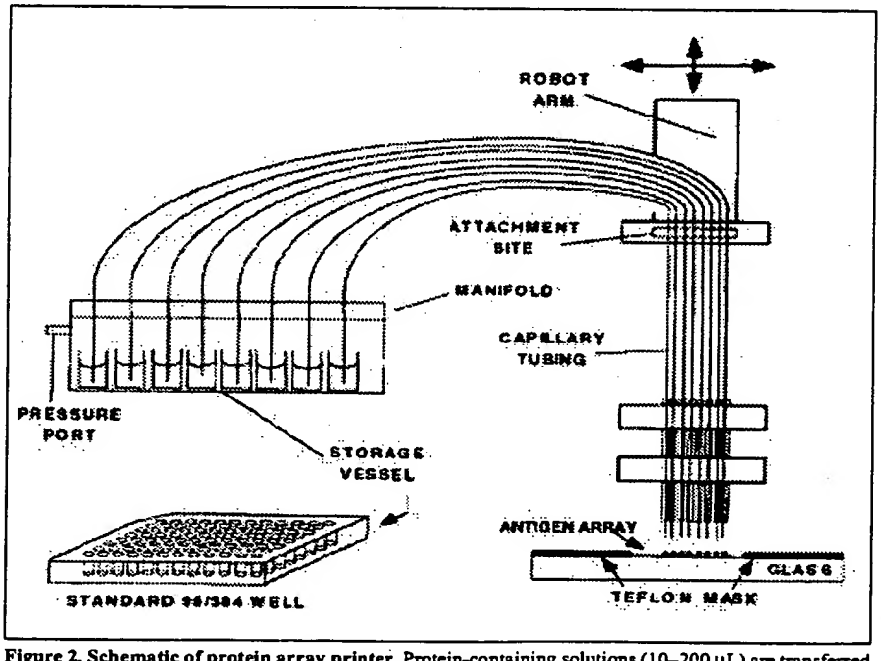


Figure 2. Schematic of protein array printer. Protein-containing solutions (10–200 µL) are transferred into wells of a standard 96- or 384-well microplate. All antigen solutions are normalized to contain the same protein concentration (i.e., equimolar). The microplate is placed in the printer manifold, and the capillaries are primed with protein solution under nitrogen pressure. After priming is complete, computer-controlled contact printing of arrays begins.

Table 2. Detection of an Entire Microarray Plate with Various Antibodies

Column Number	Rows	Antibody
Column 1	(A–H)	Anti-Guinea Pig Monoclonal
Column 2	(A–H)	Anti-Chicken Monoclonal
Column 3	(A–H)	Anti-Cat Monoclonal
Column 4	(A–H)	Anti-Rabbit Monoclonal
Column 5	(A–H)	Anti-Bovine Monoclonal
Column 6	(A–H)	Anti-Goat Monoclonal
Column 7	(A–H)	Anti-Rat Monoclonal
Column 8	(A–H)	Anti-Human Monoclonal
Column 9	(A–H)	Negative Control (No Monoclonal)
Column 10	(A–H)	Mix of All Monoclonals
Column 11	(A–H)	Mix of All Monoclonals
Column 12	(A–H)	Mix of All Monoclonals

The table details the biotinylated detection antibody or mixtures that were incubated with each well on the microarray plate. All 96 arrays on the plate were simultaneously incubated with a 1:1000 dilution of the indicated detection antibodies.

teflon-coated glass plate.

The pre-described configuration allows the source microarray sector to be excited from the bottom of the plate and fluorescence emission detected from the top of the plate by the Z-axis mounted CCD camera. A two-stage cover provides a light-tight enclosure, and an automated door allows plates to be loaded with conventional robotic arms. The scanning CCD imager is mounted on a custom mobile cart that houses the computer and stepper drive control components.

Total instrument control, including motion control and image acquisition,

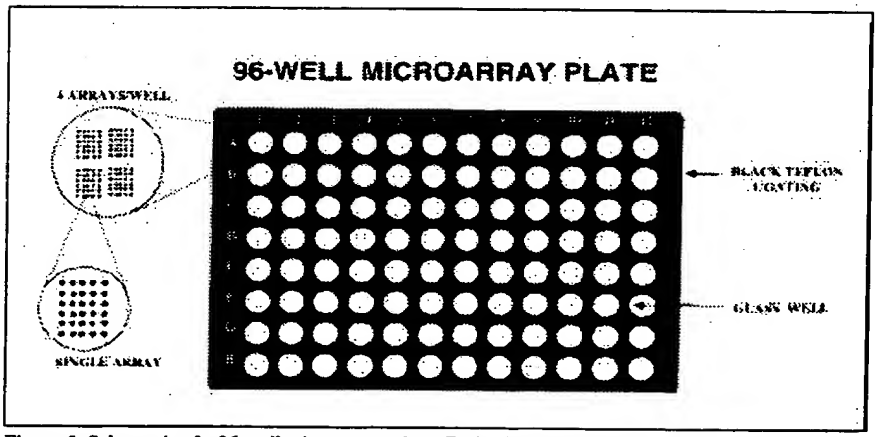


Figure 3. Schematic of a 96-well microarray plate. Each of the individual 96 glass wells contains antigens printed on its surface. Four identical 6 × 6 antigen arrays are printed in each well (144 elements per well) and serve as replicates to enhance assay validity. All arrays on the plate are identical to each other, forming a highly parallel assay format.

is provided by GeneView™ Pro software (Genometrix, The Woodlands, TX, USA), complete with database and image-analysis tools. GeneView Pro is a user-friendly visual basic, multithreaded, graphical interface that utilizes OPTIMAS™ image processing tools (Media Cybernetics, Bothell, WA, USA) and Compumotor's motion control dynamic linked libraries (DLLs). GeneView Pro allows users to select automated or single sector image acquisition. The automated scanning feature captures 4 sector images for the 96-well slides. The resulting 16-bit (2.5-MB) images are saved to disk with a standard file-naming convention. Single sectors can be imaged by manually selecting the sector of interest. Ideally, single sector imaging can be used to set and save optimized CCD integration times before initiating full-plate automated image acquisition. A full microplate (containing 96 microarrays) can be imaged in 30 s using a 5-s integration time, which includes image retrieval and file archiving.

Conventional CCD Camera

A commercially available CCD camera system was used to generate low-resolution "pan" images of an entire 96-well array plate. The AlphaImager® 2000 Digital Imaging System (Alpha Innotech, San Leandro, CA, USA) includes a light-tight enclosure containing a dual light (302/365 nm) transilluminator. The camera system consists of a high-performance CCD camera with 12.5 × 75 mm zoom lens, close-up +2 diopter lens, interference filter and a Windows®95 (Microsoft, Redmond, WA, USA) computer system (Pentium 166 MHz). Image acquisition, enhancement and analysis software are all under AlphaEase™ (Alpha Innotech) control. Microarray plates were imaged using a standard transilluminator (UV 365 nm) for 60 s.

RESULTS

Standard ELISAs immobilize antigens or antibodies by passive adsorption to a plastic substrate (polystyrene). However, passive adsorption is not an efficient method of immobilizing proteins to glass surfaces. For this reason, 96-well glass microarray plates were first amino-modified using APTS followed by surface activation using a

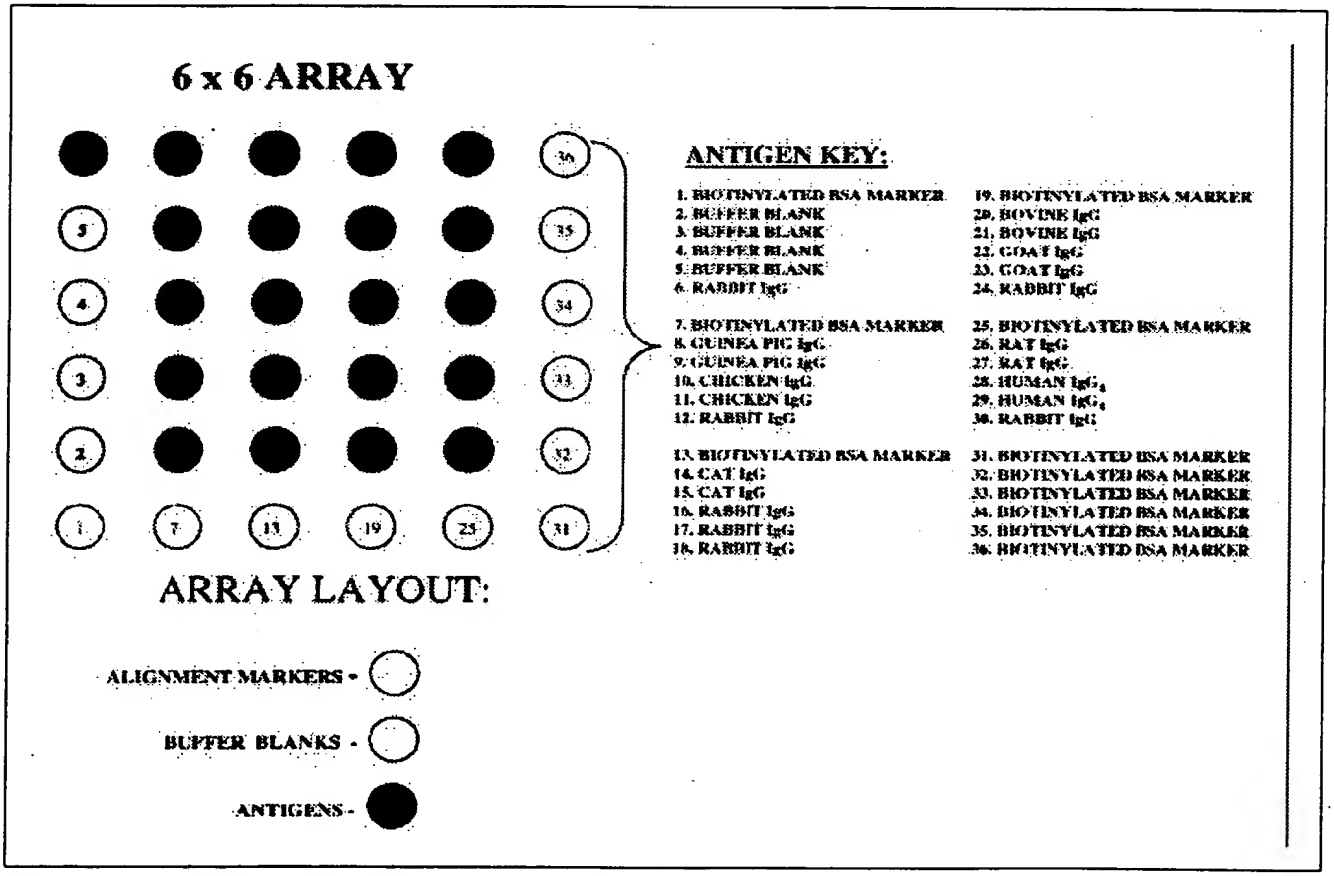


Figure 4. Array layout and antigen key. Printed antigen elements within each microarray are numbered in the layout. Numbers correspond to immobilized antigen as indicated in the antigen key. Note that some elements in the 6 × 6 array correspond to buffer blanks (carbonate buffer only). The remaining elements in the layout correspond to biotinylated markers, which permit spatial orientation of any given antigen relative to the markers.

water-soluble homobifunctional NHS-ester. This NHS-activated surface was capable of efficiently immobilizing protein-based antigens. Figure 1 illustrates the ELISA format used in these experiments.

All protein arrays were printed using the continuous flow, capillary-based printer (Figure 2). Arrayed antigens consisted of purified polyclonal IgGs isolated from various animal sources (Table 1). The antigens were contact printed onto a 96-well glass microarray plate. This microarray plate is illustrated in Figure 3. Note that four identical 6 × 6 arrays are printed in each well. This simple contact redundancy (144 elements per well) executed by the robotic printer gives strong statistical confidence in assay results generated from each 7-mm well.

Figure 4 shows the spatial layout key for the antigen array. As seen in the figure, the entire first row (array elements 1, 7, 13, 19, 25 and 31) and the last column (array elements 31–36) consist of biotinylated BSA markers. These markers allow unequivocal orientation of the array and identification of individual elements within the array. Printing of biotinylated marker proteins in each array also serve as a positive control for detection reagents (i.e., streptavidin:AP conjugate and ELF). Additionally, individual antigen elements in each array are printed in duplicate.

After printing, an entire 96-well array plate was incubated with either single or multiple monoclonal antibodies to test specificity and multiplexing characteristics. To demonstrate the specificity of each biotin-labeled monoclonal antibody for its cognate antigen, each array column (8 wells per column) on the plate was incubated, after a blocking step, with its cognate monoclonal as outlined by the incubation regimen in Table 2. Other columns were incubated with either no monoclonal (negative control) or a cocktail mixture consisting of 8 different monoclonals. Antigen-antibody complexes were then detected using a streptavidin:AP conjugate and ELF as described in Materials and Methods.

The 96-well microarray plate was imaged using two separate imaging systems. The first system consisted of a low-resolution, commercially available CCD camera and a UV transilluminator (365 nm). This low-resolution camera system allows for a single pan image of all 96 arrays to be taken as seen in Figure 5. However, individual array

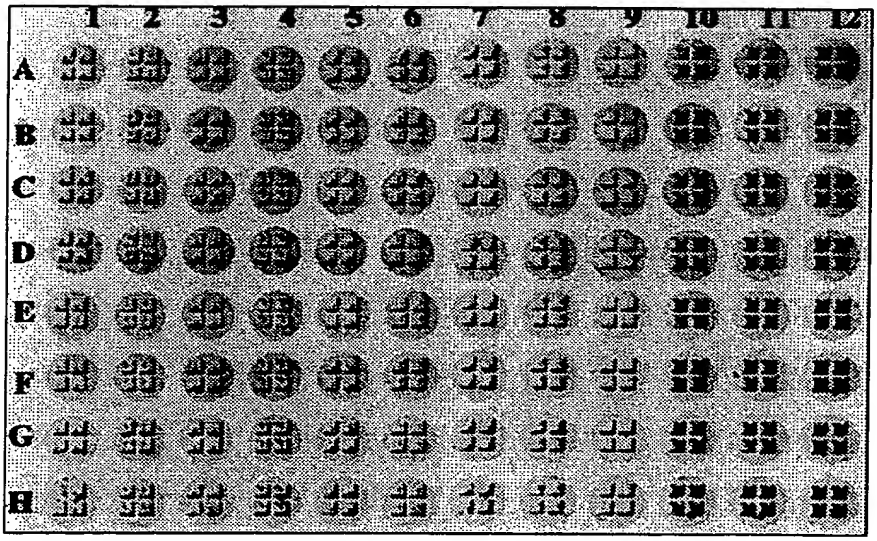


Figure 5. Low-resolution panoramic image of an entire 96-well microarray-based ELISA plate. Image was taken using a 60 exposure on the Alphalmager 2000 CCD Camera System. Array plates were excited using a standard transilluminator (UV 365) light source. Columns (1–12) and rows (A–H) use conventional microplate numbers and lettering. Each column was incubated with either single or multiple antibody mixtures as outlined in Table 2. Column 9 (A–H) consist of wells incubated with Blocker Casein in PBS only (negative controls).

elements cannot be resolved using this pan image.

To generate high-resolution images of individual elements within an array, we used the scanning CCD detector. Here, the entire plate is scanned under GeneView software control with the CCD imager taking a series of images across 4 independent sectors. Single-well images can then be magnified using the software to resolve individual elements within each array. Specificity and/or cross-reactivity of each antigen in the array for its cognate monoclonal antibody (or mixtures) was readily seen from these high-resolution CCD images (Figure 6).

A separate 96-well microarray plate was used to measure general assay sensitivity using a single monoclonal antibody. Using this new plate, arrays were incubated in parallel with various dilutions of rabbit IgG-specific monoclonal antibody. Figure 7 shows parallel images of this dilution series.

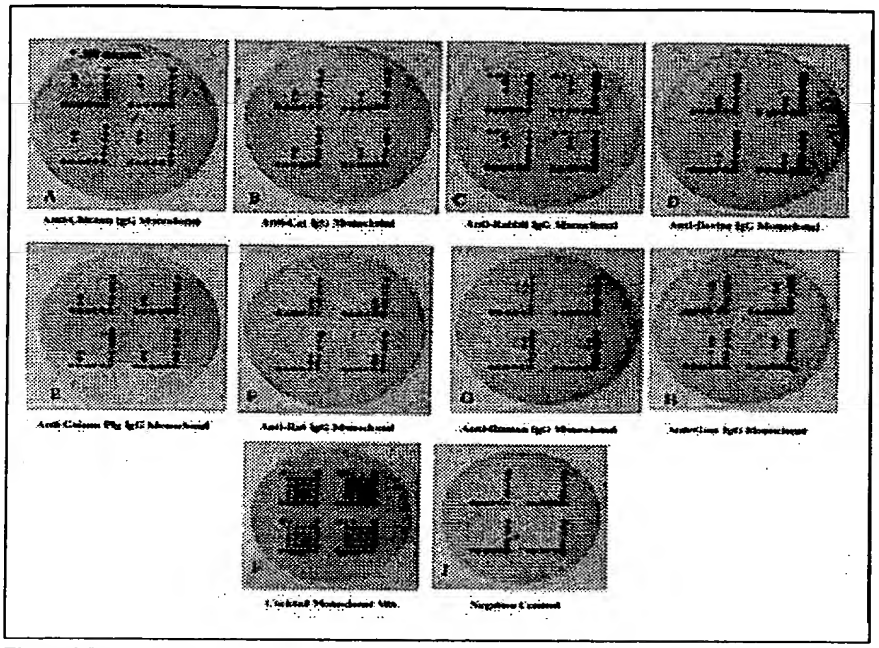


Figure 6. High-resolution CCD images of individual ELISA microarray wells. The panel consists of images A–J. All arrays were processed as indicated in the text under Microarray ELISA. Image A–H correspond to the detection of individual antigens by the indicated biotinylated monoclonal antibodies. Note that each well contains 4 identical arrays. Image I corresponds to the simultaneous detection of all antigens within the 6 × 6 array. Image J is a negative control incubated with streptavidin:AP conjugate. All images were taken using a 12-s exposure at a CCD temperature of 273 K.

DISCUSSION

This study served to evaluate the feasibility of immobilizing protein antigens on glass surfaces in a miniaturized format with a new type of array printer and specifically imaging the printed antigens in a highly parallel, multiplexed ELISA format compatible with high-throughput analysis systems. The critical element in designing a microarray-based ELISA is the potential for cross-reactivity when incubating different monoclonal or polyclonal antibodies with an array of immobilized antigens. Although monoclonal antibodies recognize a single epitope, it is possible that structurally related proteins might have similar epitopes. In these experiments, all the antigens consisted of polyclonal IgGs. Although these antigens were isolated and purified from different animal sources, they do share structural similarity. For this reason, some cross-talk between antigens was not entirely unexpected for these commercial antibodies. Even so, specificity was demonstrated using anti-guinea pig, anti-chicken, anti-cat, anti-rabbit, anti-bovine and anti-rat monoclonal antibodies for their respective antigens. However, slight cross-talk was

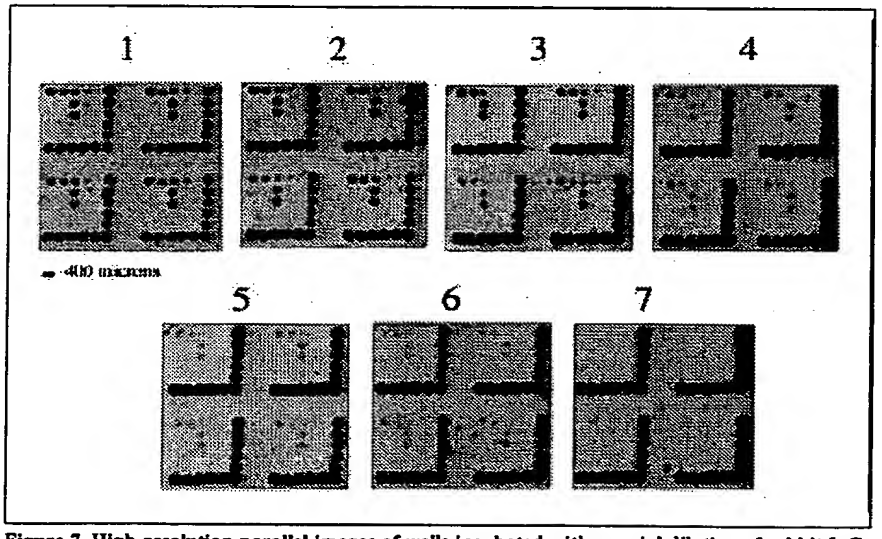


Figure 7. High-resolution parallel images of wells incubated with a serial dilution of rabbit IgG-specific monoclonal antibody. Seven parallel wells were incubated with 25-µL volumes of the indicated dilution of biotinylated rabbit IgG antibody in Blocker Casein solution or Blocker Casein only (negative control). Images 1–6 correspond to a 1:25 000, 1:50 000, 1:100 000, 1:150 000, 1:200 000 and 1:300 000 dilutions, respectively. Image 7 is the negative control. The 1:300 000 dilution corresponds to a final antibody concentration of 13.4 ng/mL. Arrays were incubated with substrate for 20 min. Images were taken using a 12-s exposure at a CCD temperature of 273 K.

observed with both anti-goat IgG and anti-human IgG monoclonal antibodies. The anti-goat monoclonal had cross-reactivity with bovine IgG, and the anti-human IgG antibody had slight cross-reactivity with goat IgG. An assay control that excluded incubation with secondary antibody but included incubation with the streptavidin:AP conjugate was negative across all arrays. These results suggest that successful multianalyte analysis using microarray-based ELISAs is strictly a matter of carefully screening each monoclonal antibody clone against all antigens on the array.

To evaluate ELISA sensitivity, a monoclonal antibody dilution series directed against rabbit IgG was used. This dilution series demonstrated that rabbit IgG antigen could easily be detected using a 1:300 000 dilution of its cognate antibody (Figure 7). In this ELISA, the original rabbit IgG monoclonal antibody stock concentration was 4.1 mg/mL. This corresponds to an assay sensitivity of 13.4 ng/mL or 340 pg of rabbit IgG monoclonal per well (25-μL total volume). ELISA sensitivity was limited by the fact that arrays were incubated with fluorescent substrate for only 5 min (i.e., far from the optimal signal generating incubation period [30–45 min] recommended by the manufacturer). Lower detection limits were compromised because these preliminary arrays were not designed for maximum assay sensitivity. Rather, they were designed primarily to test the concept of multianalyte specificity. Detection sensitivity was compromised because of excess fluorescent backgound signal emanating from the biotinylated BSA markers used to define the perimeter of each array relative to the signal generated from the specific antigen. These marker sites generate a very large signal compared to the signal generated from the 1:300 000 dilution of biotin-labeled secondary antibody for its specific cognate antigen on the array.

In retrospect, when designing future microarray ELISAs for high sensitivity, it might be preferable to use a secondary antibody for indirect detection of both markers and antigens. Indirect detection would allow signals generated from the markers to be attenuated simply by controlling the final dilution of detector antibody. This simple modification would permit long substrate incubation periods without excess background emanating from the markers. In these experiments, detection levels were not limited by detector sensitivity since the peltier-cooled CCD was capable of much longer integration times.

In summary, we have demonstrated the potential to conduct multianalyte assays using a new 96-well microarray-based ELISA format. All aspects of the process have been validated including high-speed microarray printing, antigen immobilization, ELISA specificity/sensitivity and automated microarray imaging. Monoclonal antibodies used to develop multiplexed assays must be carefully screened to avoid cross-talk between different but similar antigen sites on the array. When designing arrays, it may sometimes be necessary to screen several antibody clones for lack of cross-reactivity to other antigens on the array. Cross-reactivity on microarray-based ELISAs is analogous to cross-hybridization signals seen between single mismatch DNA capture probes and their cognate target sequences.

Advantages of this new 96-well format include both parallel and multiplexing features. Print redundancy within each well adds greatly to visual confirmation of assay validity, and the open architecture format is fully compatible with high-throughput robotic liquid handling systems and automated microarray imaging systems.

In the future, it is likely that antibody-based microarrays will be used to directly detect protein expression products from crude cell lysates. It will be interesting to validate any such arrays against cDNA-based arrays that measure expression indirectly at the mRNA level. Finally, chip-based formats promise to increase clinical diagnostic throughput in cancer and infectious disease diagnostics while simultaneously reducing cost.

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Address correspondence to:
Leopoldo G. Mendoza
Genometrix
3608 Research Forest Drive
Suite B7
The Woodlands, TX 77381, USA
Internet: lmendoza@genometrix.com